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Ultrastructure and Biochemistry of the Cell Wall of *Methanococcus voltae*

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The ultrastructure and chemical composition of the cell wall of the marine archaebacterium *Methanococcus voltae* were studied by negative-staining and freeze-etch electron microscopy and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *M. voltae* possesses a single regularly structured (RS) protein layer external to the plasma membrane. Freeze-etch preparations of cells indicated that the protein subunits are hexagonally arranged with a center-to-center spacing of approximately 10 nm. The extracted RS protein had a molecular weight of 76,000. It was present on envelopes prepared by shearing in a French press, osmotic lysis, or sonication, as indicated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. NaCl was not required for attachment of the RS protein to the underlying plasma membrane. The hexagonal array could be demonstrated by platinum shadowing and freeze-etching of envelopes, but negative staining in the absence of NaCl failed to stabilize the array. The RS protein could be solubilized by urea, guanidine hydrochloride, dithiothreitol, and several detergents, including Nonidet P-40, Triton X-100, and Tween 20. However, the most specific release of the wall protein from envelopes occurred after a heat treatment in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer at 50 to 60°C.

Methanogens, extreme halophiles, and certain thermoacidophilic and sulfur-dependent organisms compose the archaebacteria, a third line of evolutionary descent distinct from the eubacteria and eucaryotic cells (37). One of the distinguishing features of the archaebacteria is the wide variety of cell wall types, none of which possesses the typical murein structure of eubacteria (17). Archaebacterial cell walls can possess acidic polysaccharides, (*Halococcus* and *Methanosarcina* spp.), pseudomurein (*Methanobacterium* spp.), and protein sheaths (*Methanospirillum* spp.), as well as regularly-structured (RS) protein or glycoprotein layers. These protein layers are commonly referred to as RS (21) or S (surface) (32) layers. The RS layers of archaebacteria have predominantly hexagonal symmetry (33). Whereas RS layers in eubacteria form a surface layer external to the wall, in the archaebacteria (including thermoacidophilic, halophilic, and methanogenic organisms), they may be the sole component of the wall (32, 33).

Among the methanogens, there are RS layers in numerous genera including *Methanococcus*, *Methanolobus*, *Methanothermus*, *Methanoplanus*, *Methanomicrobium*, and *Methanogenium* (18, 32, 33). Most reports are limited to observations with the electron microscope of structured arrays visualized on the cell surface after negative staining or freeze-etching. In archaebacteria, virtually nothing is known about the biochemistry of RS layers or the assembly, secretion, and attachment of the protein to the plasma membrane or wall. In this paper, we report the beginning of a detailed study of the RS layer of the marine methanogen *Methanococcus voltae*. This organism was chosen for study because its nutritional requirements are well known and growth can be obtained in a defined medium (36). Furthermore, it is favored for those beginning genetic work on the methanogens (38) and it has been the object of considerable research dealing with transport (14), bioenergetics (15), and biosynthetic pathways (9), which could prove useful for further investigations.

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MATERIALS AND METHODS

Organism and growth conditions. *M. voltae* PS, obtained from L. Hook, Ohio State University, Columbus, was grown with gentle shaking in 100 ml of either Balch medium III (1) or the defined medium of Whitman et al. (36) in 1-liter bottles modified to accept serum stoppers. The gas atmosphere was CO_2-H_2 (1:4, vol/vol). Occasionally, cells were grown in a complex medium in an Airlift Fermentor (Bethesda Research Laboratories, Inc., Gaithersburg, Md.).

Isolation of cell envelopes. Cell envelopes were prepared from *M. voltae* by sonication, shearing in a French press, or osmotic shock. For envelopes prepared by using a French press or sonication, cells were harvested by centrifugation (8,000 × *g* for 10 min), suspended in a small volume of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.2) containing 2% NaCl (HEPES-Na buffer), and stored on ice. The cells were either broken by a single pass through a French pressure cell (American Instrument Co.) at 138,000 kPa or sonicated by three 1-min bursts on ice with a Virsonic Cell Disrupter (The VirTis Co., Inc., Gardiner, N.Y.) operated with the intermediate tip at 50% maximum. The product of each disruption method was treated with DNase to reduce the viscosity and centrifuged at 3,000 × *g* for 10 min to remove any remaining intact cells. The supernatant was subsequently centrifuged at 40,000 × *g* for 30 min to pellet the envelopes. The envelope fraction was washed once in HEPES-Na buffer and stored as a suspension in HEPES-Na buffer at −20°C. Envelopes from osmotically lysed cells were prepared by suspending the harvested cells in the defined growth medium of Whitman et al. (36) without the NaCl and $NaHCO_3$ (lysis buffer). The cells lysed immediately, and after the viscosity was reduced by the

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addition of DNase, the envelopes were isolated by centrifugation as described above.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (23). Electrophoresis was performed in slabs 1.5 mm thick and 15 cm long with a 4.5% stacking gel over a 12.5% running gel. Samples were electrophoresed at room temperature at 100 mV until the sample entered the running gel and then at 150 mV. The gels were stained for 1 h at 60°C in 0.2% Coomassie brilliant blue R250 and destained by diffusion.

Extraction of cell envelopes. Cell envelopes prepared by osmotic lysis were treated with a variety of salts, detergents, and chemical perturbants in attempts to obtain specific release of the RS layer. The following reagents were used: 0.5% Triton X-100, 0.5% Sarkosyl (CIBA-GEIGY Corp.), 0.5% sodium deoxycholate, 0.5% Tween 80, 0.5% Nonidet P-40 (NP-40), 0.5% sodium dodecyl sulfate, 0.5% Brij 58, 0.5% Triton X-405, 0.5% Triton X-165, 0.5% Triton N-101, 0.5% Triton X-114, 0.5% Tween 20, 20 mM lithium 3,5-diiodosalicylate (LIS), 50 mM dithiothreitol, 10 mM EDTA, 3 M NaCl, 3 M LiCl, 3 M $MgCl_2$, 8 M urea, and 6 M guanidine hydrochloride. The envelopes were suspended to 1.5 mg/ml in 10 mM HEPES-Na buffer containing the agent to be tested. Incubations were at 37°C for 1 h, followed by centrifugation at 40,000 × *g* for 30 min and one wash of the pellet in HEPES-Na buffer. Since some of the detergents were incompatible with 2% NaCl, detergent extractions were repeated in the absence of salt.

Preparation of specimens for electron microscopy. (i) Freeze-etching. To prepare samples for freeze fracture and etching, cells were grown in 10 ml of Balch medium III and then harvested by centrifugation (8,000 × *g* for 10 min at 4°C). A small amount of wet, packed, unwashed cells was placed onto the center of a gold grid, immediately frozen in liquid Freon 22 (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) for 1 min, and stored in liquid nitrogen until used. Cryogens were not used. Cleaving, etching, platinum-carbon shadowing, and replication were done in a Balzers freeze-etch apparatus (model BA510M; Balzers AG, Liechtenstein). Samples were etched for 2 min, and shadowed and carboned for 5 s each, and the replica was floated off onto distilled water. The replica was cleaned successively in acid hypochlorite (Javex)-distilled water-concentrated sulfuric acid-distilled water-Javex, and distilled water. The replica was picked up on a Formvar-coated 200-mesh copper grid and air dried before examination.

(ii) Negative staining. Samples were negatively stained and supported on carbon-Formvar-coated copper grids. A 400-mesh grid was inverted over a drop of the sample for 1 min and subsequently stained on a drop of 2% ammonium molybdate (pH 7).

(iii) Platinum shadowing. A 400-mesh carbon-Formvar-coated copper grid was inverted over a drop of the sample for 1 min, and the excess fluid was removed by touching the edge of the grid to a torn piece of filter paper. The grid was air dried, and the specimen was shadowed with platinum at a 45° angle by using the Balzers freeze-etch apparatus described above.

(iv) Fixation, embedding, and staining techniques. Cells were fixed in 2% paraformaldehyde–3% glutaraldehyde–1% acrolein–2.5% dimethyl sulfoxide in 0.05 M cacodylic buffer (pH 7). The cells were prefixed by addition of the fixative to give a 1/10 concentration immediately upon opening of anaerobic bottles. After 5 min at room temperature the cells were centrifuged and suspended in the fixative containing 2% NaCl. After 30 min at room temperature, samples were fixed at 4°C for 24 h. The samples were enrobed in 2% Noble agar (Difco Laboratories), and blocks were postfixed at room temperature for 1 h with 1% OsO_4–2% NaCl and for 2 h with 1% uranyl acetate–2% NaCl. The blocks were dehydrated in an acetone series and embedded in Vestopal W (Martin Jaeger Co., Geneva, Switzerland). Sections were cut and stained with uranyl acetate and lead citrate.

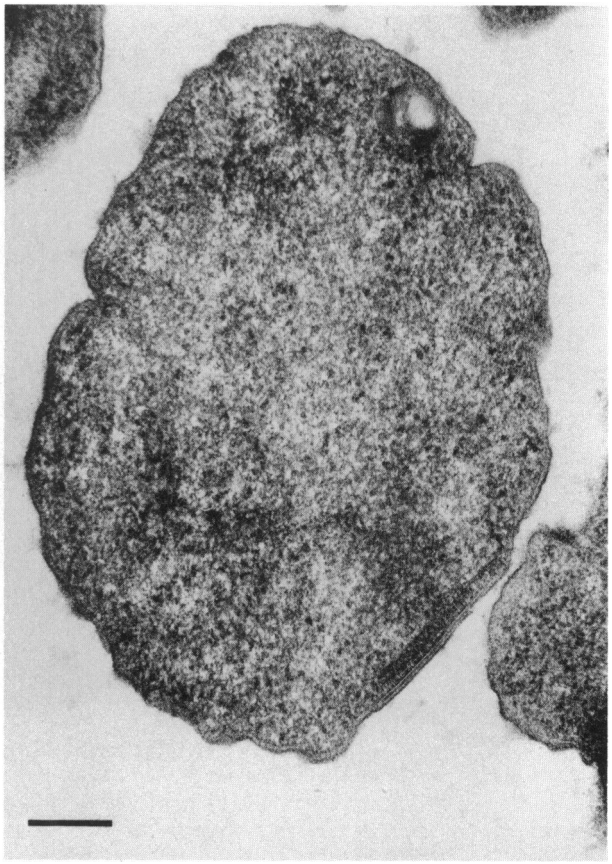

FIG. 1. Thin section of *M. voltae*. Bar = 200 nm.

Electron microscopy. Specimens were examined in a Philips EM 300 electron microscope operating at 60 kV. Micrographs were taken on fine-grain positive film (Eastman Kodak Co., Rochester, N.Y.).

Chemicals. All detergents were purchased from Sigma Chemical Co., St. Louis, Mo. except Tween 80, which was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. Electrophoresis chemicals were from Bio-Rad Laboratories, Mississauga, Ontario, Canada, and lipase (*Rhizopus arrhizus*) was from Boehringer Mannhein Biochemicals, Dorval, Quebec, Canada.

RESULTS

Ultrastructure. *M. voltae* is a regular- to irregular-shaped coccus (Fig. 1) with tufts of flagella and a few fimbriae (Fig. 2). Thin sections of cells grown in either Balch III or defined medium showed that the cell envelope consisted of a single layer of wall material external to the plasma membrane (Fig. 3A). The outer edges of the wall layer appeared to stain more densely. The periodicity of the protein subunits composing this wall layer was best seen in freeze-etch replicas of whole

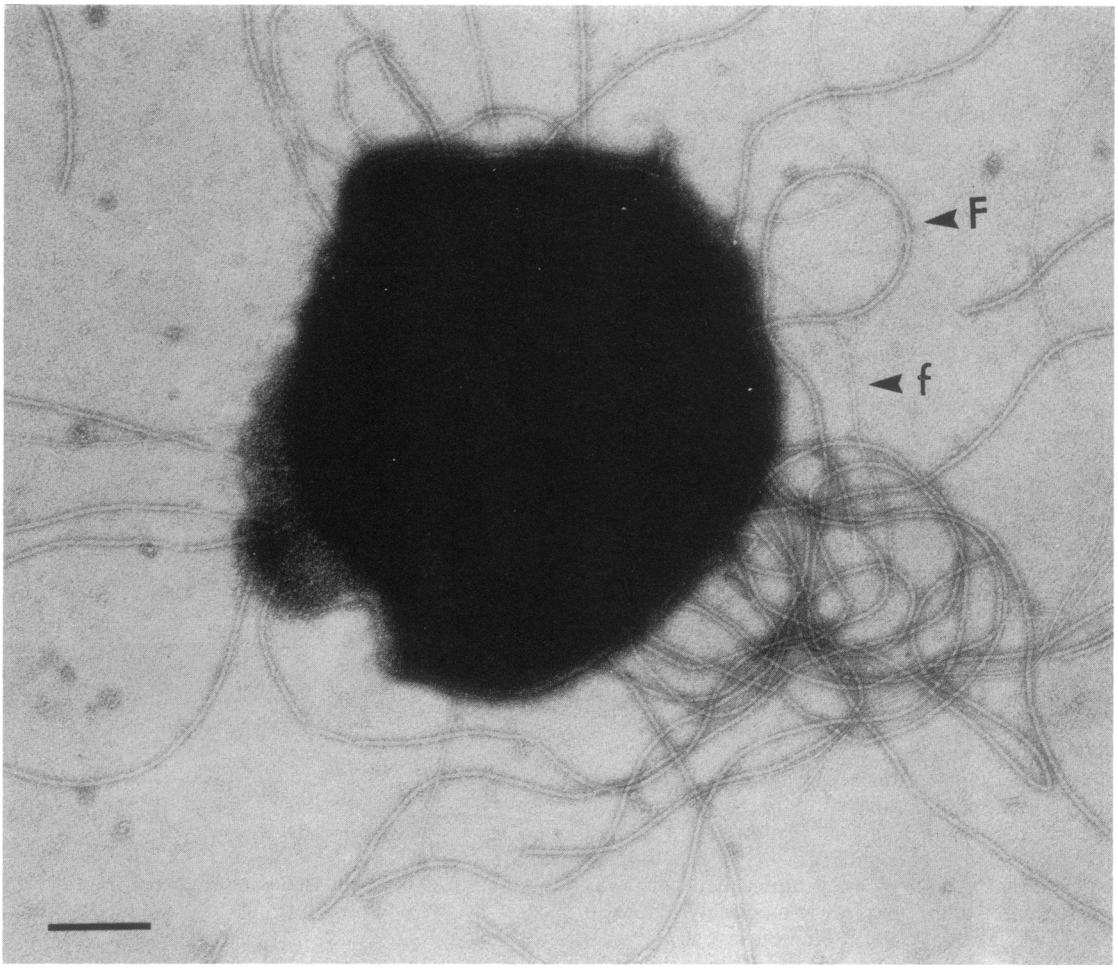

FIG. 2. Negatively stained (1% ammonium molybdate) preparation of a *M. voltae* cell which shows the attached flagella (F) and fimbriae (f). Bar = 200 nm.

cells (Fig. 4A). The RS layer was hexagonally arranged with a center-to-center spacing of approximately 10 nm. One fracture plane was observed in the entire envelope, and this passed through the hydrophobic region of the plasma membrane (Fig. 4B). No fracture plane was observed within the wall.

M. voltae lyses easily in sodium-deficient medium or buffer to produce large envelope fragments that are suitable for ultrastructural and biochemical analyses. Cell envelopes prepared by osmotic lysis of *M. voltae* grown in either complex or defined medium gave very similar protein profiles when examined by SDS-PAGE on 12.5% gels (Fig. 5). The major band, the RS protein, had a molecular weight estimated at 76,000, identical to the unpublished value of Konig and Stetter reported in a recent review (32). Other prominent envelope proteins migrated with molecular weights of 60,000, 58,000, 44,000, 32,000, 17,000, 15,500, and 14,000. Negative staining (with ammonium molybdate, uranyl acetate, or sodium zirconium glycolate) of envelopes prepared either by osmotic lysis in NaCl-deficient medium or 10 mM HEPES buffer or sonication in 10 mM HEPES buffer did not reveal an RS wall layer (Fig. 6A). Some alignment and periodicity could be seen in this and other images, but the hexagonal arrangement of subunits was poorly preserved. However, the wall layer could be seen on the edge of envelope fragments (Fig. 6A), and SDS-PAGE of these envelopes showed that the wall protein was present (data not shown). To test the hypothesis that sodium may be required for structural stability of the RS layer, envelopes prepared in the presence and absence of 2% NaCl were examined by platinum shadowing and freeze-etching. These techniques were used because the presence of 2% NaCl is incompatible with the negative-staining technique. The hexagonal surface array was seen on both preparations of envelopes with both techniques (Fig. 6B and 7). The reason for the lack of success with the negative-staining technique remains obscure.

Thin sections of cells grown in either Balch III or defined medium also revealed the presence of an unusual structure underlying regions of the plasma membrane. An electron-dense band lined the plasma membrane at a distance of 25.5 to 28.5 nm (Fig. 3B). Diffuse striations were present in the space between this band and the plasma membrane. An additional inner electron-dense bilayer was present which extended the entire length of the structure. It was not always present at a uniform distance from the plasma membrane and often appeared wavy. This entire structure was often found adjacent to flagellar insertion sites. This structure is reminiscent of the polar membrane first observed in *Aquaspirillum serpens* by Murray and Birch-Andersen (28).

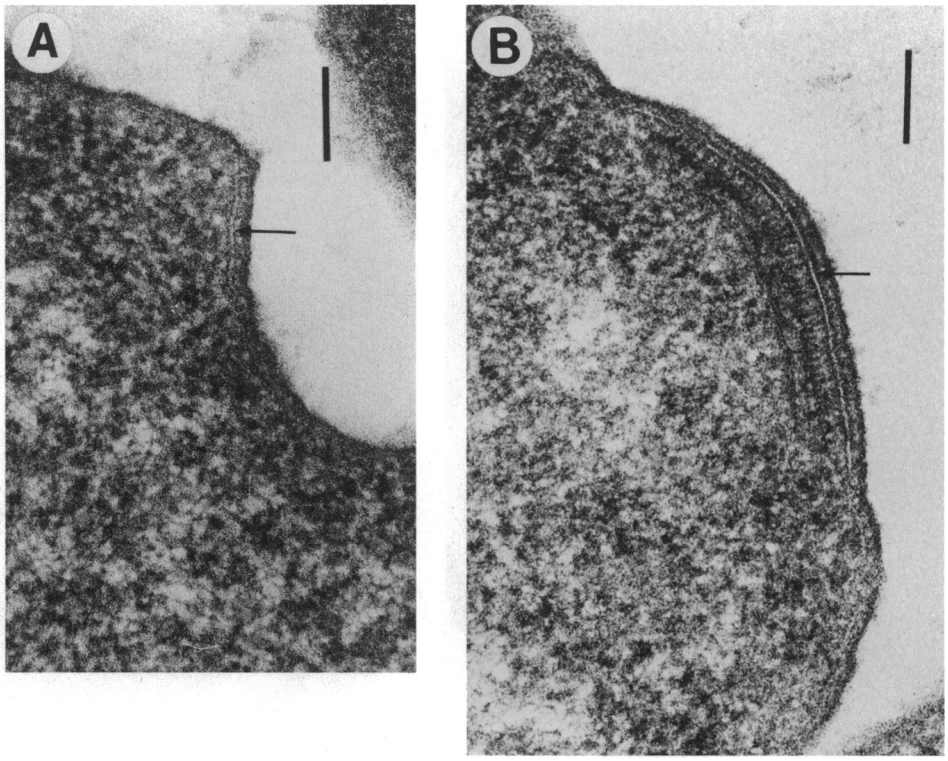

FIG. 3. Thin sections of *M. voltae* which show cell envelope profile (A) and polar membrane-like structure underlying the plasma membrane (arrow) (B). Bar = 100 nm.

Solubilization of the cell wall protein. Initially, envelopes prepared by osmotic lysis were suspended in HEPES buffer (pH 7.5) with or without 2% NaCl and heated at 25, 37, 50, or 60°C for 1 h, followed by centrifugation at 40,000 × *g* for 30 min. Examination of the pellets by SDS-PAGE revealed a preferential release of the wall protein at 50°C in the presence of NaCl, whereas a higher temperature (60°C) was required for release in the absence of NaCl (Fig. 8). Examination of lyophilized supernatant material by SDS-PAGE revealed a preferential solubilization of the wall protein. The preparation also contained a small amount of membrane proteins but could be enriched for the wall protein by treatment of the supernatant with 1 M guanidine hydrochloride for 1 h at 37°C followed by centrifugation at 80,000 × *g* for 1 h. This supernatant was dialyzed and concentrated in an Amicon filtration cell (Amicon Corp., Lexington, Mass.) with a PM 10 filter, and when examined by SDS-PAGE, this material showed very little contamination with other membrane proteins (Fig. 9). Attempts were also made to specifically extract the RS protein from isolated envelopes by using a variety of salts, detergents, and other membrane perturbants. Extraction of envelopes with high salt concentrations and EDTA did not solubilize the wall protein (Fig. 10). The wall protein was removed by dithiothreitol and the hydrogen bond-breaking reagents guanidine hydrochloride and urea. At least 4 M urea or 1 M guanidine hydrochloride was required to release the wall protein. Identical results were obtained with these compounds regardless of the presence or absence of 2% (wt/vol) NaCl. The detergents NP-40, Triton X-100, SDS, Sarkosyl, Brij 58, Triton X-165, Triton X-114, Triton N-101, Tween 20, and deoxycholate all solubilized the wall protein but also resulted in the extraction of several other envelope proteins (Fig. 11). Of these detergents, NP-40, Triton X-100, Triton N-101, Triton X-114, and Tween 20 were the most specific. Tween 80, Triton X-100, and LIS had little effect on the protein profile of the treated envelopes. The presence of 2% (wt/vol) NaCl was incompatible with some of the detergents so the results are shown only for extraction in the absence of salt. The wall protein was not selectively solubilized by extremes of pH (results not shown). It was also determined that treatment of envelopes with lipase (*R. arrhizus*) released the wall protein, but in conjunction with numerous membrane proteins. Secretion of the wall protein during growth in complex medium was also examined. After removal of cells by centrifugation (10,000 × *g* for 10 min), the culture supernatant was further centrifuged (80,000 × *g* for 1 h), and this supernatant was dialyzed extensively, lyophilized, and examined by SDS-PAGE. No significant amount of wall protein was observed (data not shown).

DISCUSSION

Our results confirm the observations of Konig and Stetter that *M. voltae* has an RS protein wall layer (32). We found that this RS layer is hexagonally packed, with a center-to-center spacing of 10 nm. The RS layers of other *Methanococcus* species are also hexagonally packed (33). The freeze-fracture face observed in the plasma membrane of *M. voltae* is similar to that reported for *Methanococcus vannielii* (16). The protoplasmic fracture face of the plasma membrane of both organisms contains a dense array of intramembrane particles. Doddema et al. (8) reported that methanococci possess flagella but no fimbriae. Our observations and those of Jones et al. (16) clearly showed that both *M. voltae* and *M. vannielii* possess flagella and fimbriae.

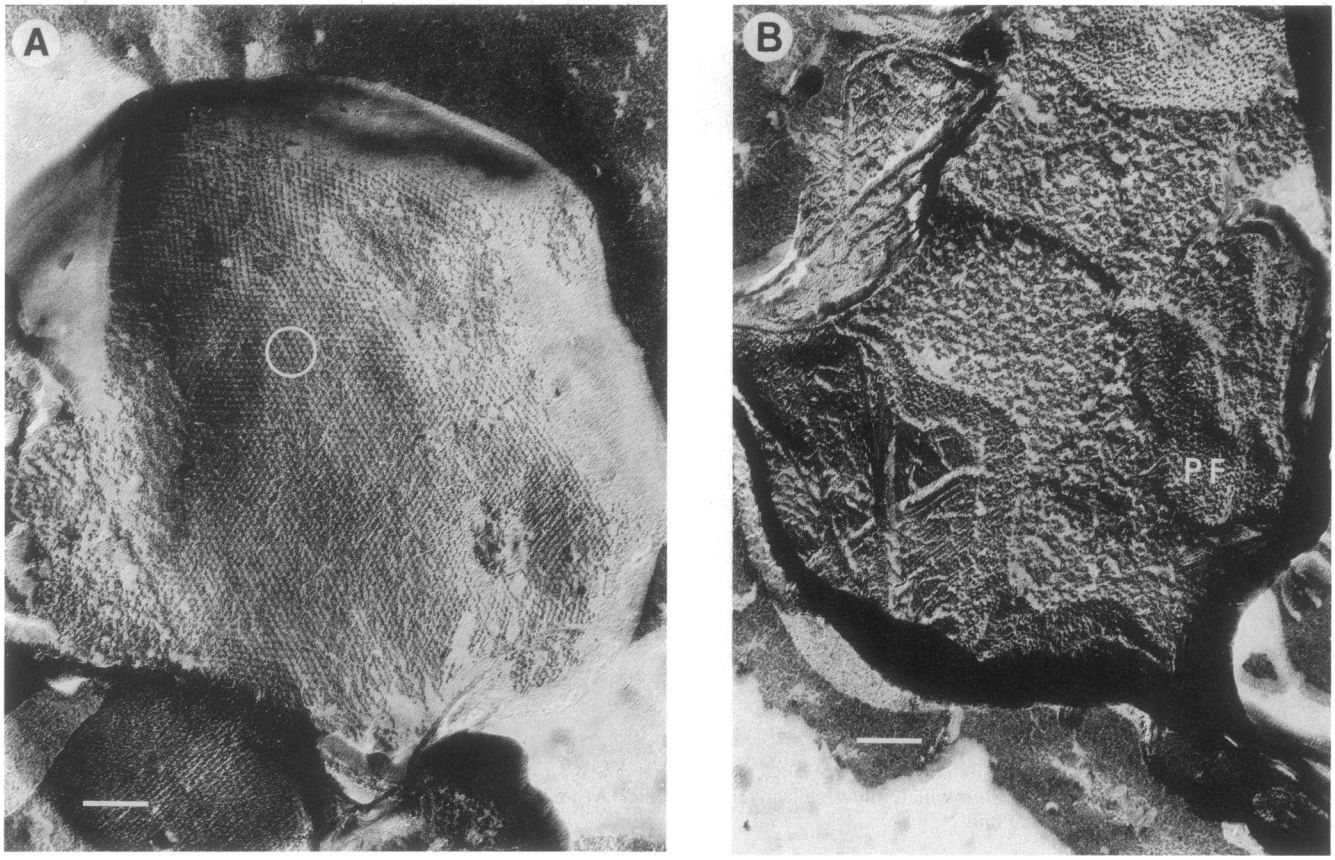

FIG. 4. Freeze-etch replicas of *M. voltae* cells which show regular hexagonal arrangement of wall protein (A) and fracture plane through the plasma membrane (B). The protoplasmic fracture face (PF) contains a dense array of intramembrane particles. The circle shows hexagonal arrangement of subunits. Bar = 100 nm.

The cell envelope profile of *M. voltae* is similar to that of the extreme halophile "*Halobacterium halobium*," now reclassified as *H. salinarium* (25). Both organisms possess a plasma membrane and one wall layer consisting of hexagonally packed protein subunits. However, the role of sodium in the stabilization of the plasma membranes is quite different. *M. voltae* grows optimally in 0.4 M (2%) NaCl. *H. salinarium* grows best in 3 to 4 M (17 to 23%) NaCl (22). *M. voltae* cells lyse in the absence of sodium, but the plasma membrane forms stable vesicles and the RS-layer protein is not solubilized from the envelope. The plasma membrane of *H. salinarium* cannot be isolated by osmotic shock since the membrane itself dissolves at decreased NaCl concentrations (5). This dissolution of the membrane can be prevented by high concentrations of monovalent cations or relatively low concentrations of divalent cations. However, the concentration of divalent cations which is sufficient to stabilize the membrane against dissolution is insufficient to stabilize the organism against osmotic lysis. The different effect of NaCl on the plasma membrane integrity of these organisms is probably a result of differences in the polar lipid composition of the membrane. The polar lipids of *H. salinarium* are very acidic and consist of a phosphatidylglycerol phosphate analog (65%) and a sulfated glycolipid (25%) (24). NaCl is

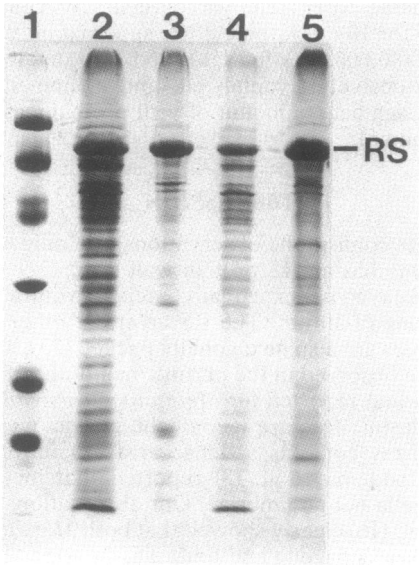

FIG. 5. SDS-PAGE of *M. voltae* cells and envelopes. Cells were grown in either complex Balch medium III (1) or the defined medium of Whitman et al. (36). Envelopes were prepared by osmotic shock. Lane 1, Bio-Rad molecular weight standards (lysozyme, 14,400; soybean trypsin inhibitor, 21,500; carbonic anhydrase, 31,000; ovalbumin, 45,000; bovine serum albumin, 66,200; phosphorylase *b*, 92,500); lane 2, cells from complex medium (100 μg); lane 3, envelopes from complex medium (70 μg); lane 4, cells from defined medium (70 μg); lane 5, envelopes from defined medium (80 μg).

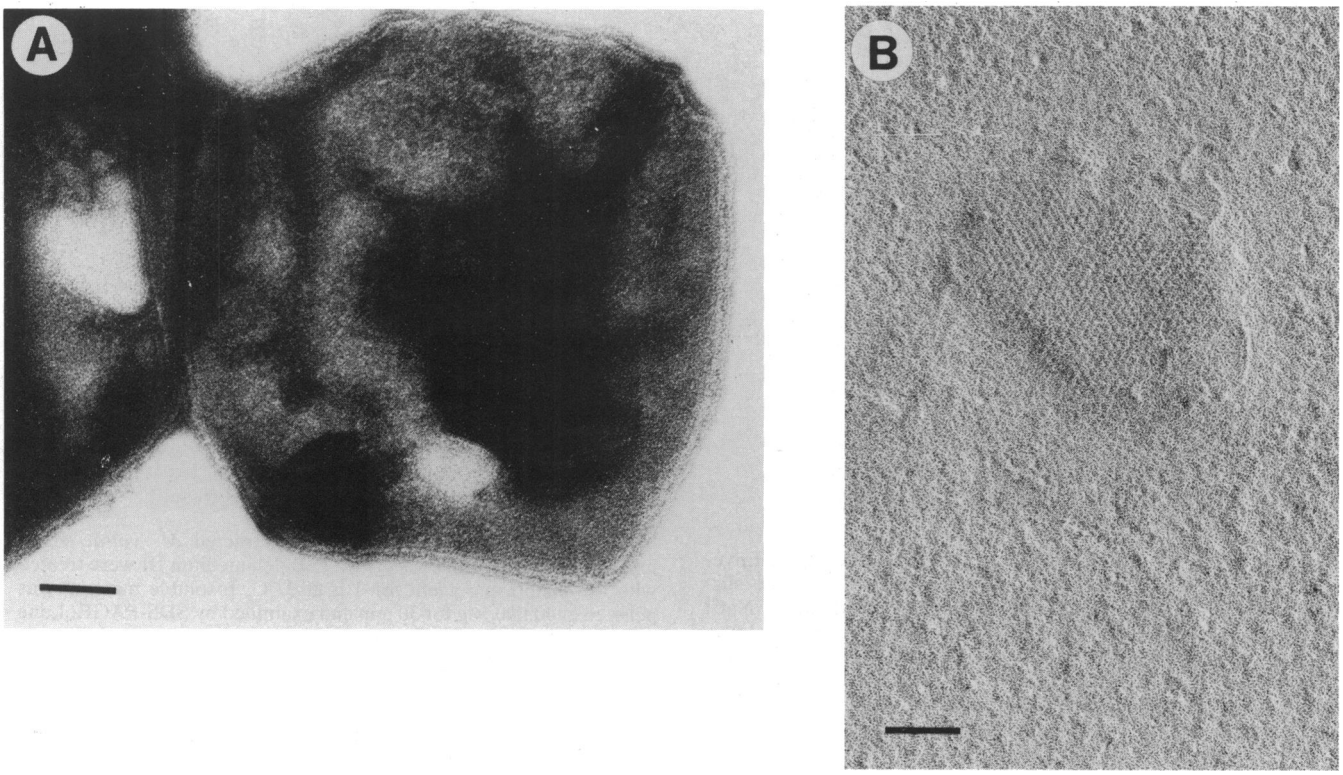

FIG. 6. Envelopes of *M. voltae* prepared by osmotic lysis in NaCl-deficient medium and negatively stained with 2% ammonium molybdate (A) or by sonication in HEPES-Na buffer and platinum shadowed (B). Bar = 100 nm.

required to neutralize the high concentration of negative charges in the membrane. The major polar lipid of *M. voltae* is a diglucosylglycerolipid (62%) (D. Sprott, personal communication).

The RS layer of *H. salinarium* is also dependent on NaCl for structural integrity. The hexagonal pattern is observed in shadowed preparations of membranes in 5 M NaCl but not in 2.5 M NaCl (4, 34, 35). The RS layer of *M. voltae* does not appear to depend on NaCl for structural stability, since the hexagonal array was seen in shadowed and freeze-etched preparations of membranes under sodium-deficient conditions. We tried a variety of negative stains to obtain a negatively stained image in which the paracrystalline arrangement of the protein subunits was well preserved, but all attempts were unsuccessful. Some cation-dependent RS layers, such as those found in *Aquaspirillum* species, are not very stable and are structurally disrupted if 1 mM $CaCl_2$ is not included in the negative stain (3). We could not use this approach since 2% NaCl is incompatible with the negative-staining technique. Messner et al. (27) reported that RS layers of many *Bacillus stearothermophilus* strains that were clearly visible in freeze-etch preparations frequently had a random granular appearance in negatively stained images. They advise prefixing cells with glutaraldehyde before negative staining. We found that the RS layer of *M. voltae* could not be demonstrated by negative staining of envelopes fixed in 2% glutaraldehyde. In contrast to the wall protein of *M. voltae*, the RS-layer protein of *H. salinarium* is solubilized when the NaCl concentration is decreased from 4.3 to 2.2 M (34). It is difficult to interpret earlier work on the wall protein of *H. salinarium*, since solubilization of the protein was not followed by SDS-PAGE. However, it appears that loss of

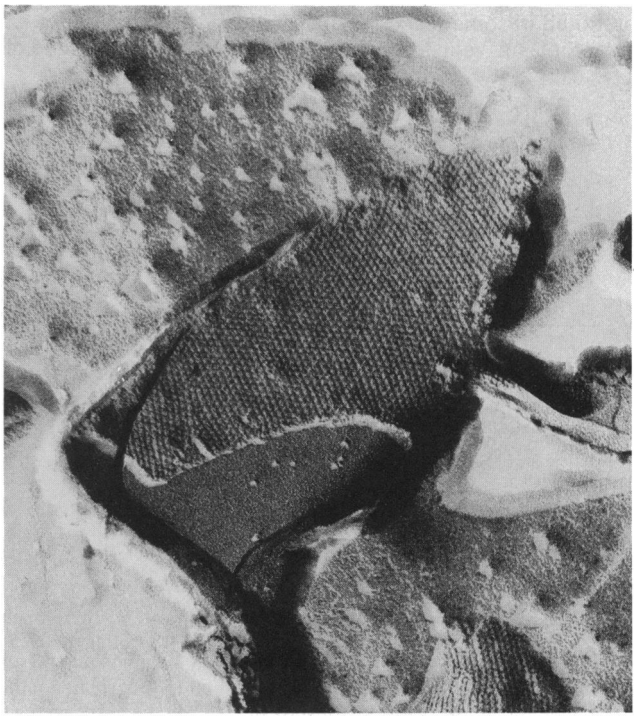

FIG. 7. Freeze-etch replica of *M. voltae* envelope prepared by osmotic lysis in distilled water. Magnification, ×85,500.

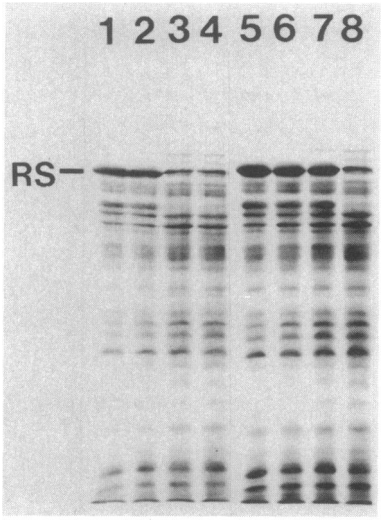

FIG. 8. SDS-PAGE of heat-treated *M. voltae* envelopes. Envelopes prepared from cells grown in Balch medium III were suspended in 50 mM HEPES buffer (pH 7.5) with or without 2% NaCl and incubated for 1 h at 20, 37, 50, or 60°C. Insoluble material was pelleted at 40,000 × *g* for 30 min and examined by SDS-PAGE. Lane 1, 2% NaCl (20°C); lane 2, 2% NaCl (37°C); lane 3, 2% NaCl (50°C); lane 4, 2% NaCl (60°C); lane 5, no NaCl (20°C); lane 6, no NaCl (37°C); lane 7, no NaCl (50°C); lane 8, no NaCl (60°C).

the hexagonal surface pattern occurs concomitantly with release of the protein from the plasma membrane. At lower NaCl concentrations, the plasma membrane begins to dissolve. Therefore, the cell wall and membrane begin to lyse at different salt concentrations and occur independently of each other (35).

The observations of a polar membrane-like structure in *M. voltae* were not entirely unexpected since Jones et al. (16) had noted in some places that a "highly structured region of the cytoplasm" existed adjacent to the plasma membrane of

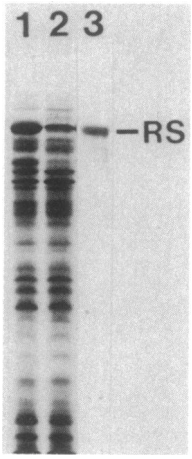

FIG. 9. SDS-PAGE of partially purified wall protein. Envelopes (lane 1) were heat treated at 50°C in 50 mM HEPES buffer (pH 7.5) plus 2% NaCl for 1 h, followed by centrifugation at 40,000 × *g* for 30 min; the pellet is shown in lane 2. The supernatant was treated with 1 M guanidine hydrochloride for 1 h at 37°C and centrifuged at 80,000 × *g* for 1 h. The supernatant (lane 3) was dialyzed in an Amicon filtration unit with a PM 10 filter before analysis.

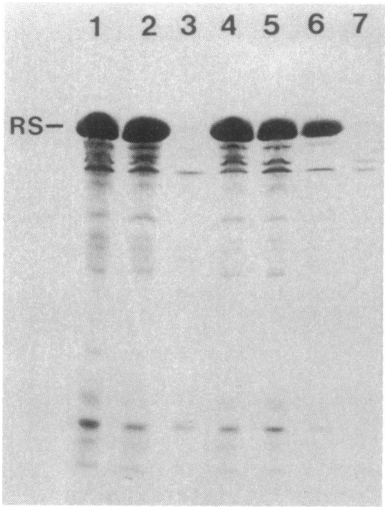

FIG. 10. SDS-PAGE of chaotrope-extracted *M. voltae* envelopes. Envelopes from cells grown in Balch medium III were treated with the chaotropic agent for 1 h at 37°C. Insoluble material was pelleted at 40,000 × *g* for 30 min and examined by SDS-PAGE. Lane 1, control; lane 2, 3 M LiCl; lane 3, 8 M urea; lane 4, 3 M NaCl; lane 5, 10 mM EDTA; lane 6, 3 M $MgCl_2$; lane 7, 50 mM dithiothreitol.

M. vannielii. However, the investigators may not have been familiar with the polar membrane structure which is often found in polarly flagellated gram-negative bacteria (*A. serpens* [28]; *Campylobacter fetus* [10, 30]; *Ectothiorhodospira mobilis* [29]; *Rhodospirillum* spp. [7, 12, 13]; *Vibrio cholerae* [10]). A polar membrane has also been observed in *Selenomonas ruminantium* (6). Typically, a polar membrane consists of a barlike array of stalks and knobs arising from the inner surface of the plasma membrane

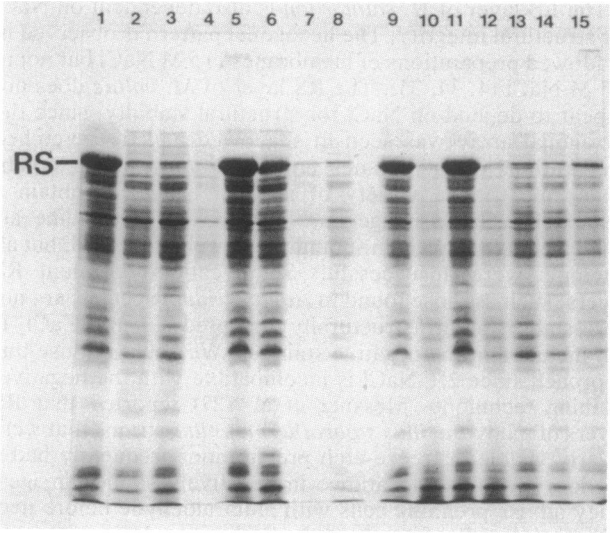

FIG. 11. SDS-PAGE of detergent-extracted *M. voltae* envelopes. Envelopes prepared from cells grown in Balch medium III were treated with detergents for 1 h at 37°C. Insoluble material was pelleted at 40,000 × *g* for 30 min and examined by SDS-PAGE. Lanes 1 and 9, control; lane 2, NP-40; lane 3, Triton X-100; lane 4, SDS; lane 5, Tween 80; lane 6, LIS; lane 7, Sarkosyl; lane 8, deoxycholate; lane 10, Brij 58; lane 11, Triton X-405; lane 12, Triton X-165; lane 13, Tween 20; lane 14, Triton N-101; lane 15, Triton X-114.

and extending into the cytoplasm approximately 20 nm. This structure has usually but not exclusively been found associated with the flagellar insertion point and near the site of septum formation. This localization of the structure has led to the hypothesis that polar membranes are involved in energy transductions (28). The structure observed in *M. voltae* generally agrees with the above description but appears to be slightly more complex, with the addition of a second electron-dense bilayer. Recently, Robertson et al. (31) have observed another type of specialized membrane structure in thin sections of *H. salinarium*. This subplasmalemma sheetlike periodically cross-banded structure is approximately 27 nm thick and extends 0.35 to 0.8 μm along the length of the plasma membrane. It was suggested that this may be a cytoplasmic appendage of the purple membrane.

Attempts to specifically extract the wall protein of *M. voltae* by the conventional methods used for eubacteria (21) were not successful. When solubilization of the wall protein occurred, as in the presence of urea, guanidinium, dithiothreitol, and some detergents, some plasma membrane proteins were also extracted. These results may reflect the different structure of this type 3 archaebacterial wall (17), in which the wall protein interacts directly with the plasma membrane without the benefit of an intervening wall layer. Eubacterial plasma membrane proteins are readily solubilized by nonionic and ionic detergents (11), and based on our data it appears that proteins embedded in the ether-linked glycerolipids of the archaebacterial membrane are likewise solubilized. The concentrations of urea and guanidinium used also solubilized some membrane proteins of *M. voltae*. It was anticipated that detergents such as Triton X-100 would solubilize the plasma membrane. Negative-staining electron microscopy of the Triton X-100-insoluble pellet of *M. voltae* envelopes showed only amorphous aggregates (data not shown). We had hoped that the wall layer would be left intact after detergent treatment, since RS layers of some eubacteria are not dissociated by nonionic detergents (2, 19). However, the wall layer of *M. voltae* was equally sensitive to the detergents. This may indicate relatively weak interactions among the units composing the RS layer. A variety of nonionic detergents with a range of hydrophile-lipophile balance (HLB) values (11) were used, but no specific release of wall protein was observed. Furthermore, no correlation between HLB values and effectiveness in solubilizing the wall protein was noted. NP-40, Triton X-100, and Triton N-101 (HLB values, 13.1 to 13.5) were effective, as was Tween 20 (HLB value, 16.7). However, Tween 80 with an HLB value of 15.0 had little effect on the solubilization of the wall proteins. LIS is a peptide-solubilizing agent that selectively solubilizes extrinsic polypeptides from erythrocyte membranes and intact viruses (26). It was effective in removing the RS-layer protein of *A. serpens* VHA (20) but not *M. voltae*.

Divalent cations may not be involved in the wall-membrane interaction, since EDTA did not release the wall protein. Hydrogen bonds may be involved in this interaction, since the protein was released with moderate concentrations of urea and guanidine hydrochloride. RS-layer subunits are noncovalently linked to each other, as well as to the underlying cell wall component, in both gram-positive and gram-negative eubacteria, and this appears true for the wall protein of *M. voltae* as well. A combination of weak bonds (hydrogen, ionic, and hydrophobic) seems to be responsible for both the structural integrity of the RS layer and its interaction with the underlying layer (32).

A promising starting point for the purification of the wall protein appears to be a mild heat treatment which preferentially removes the wall protein from the envelope.

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